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Platelet-activating factor, tumor necrosis factor, hypoxia and necrotizing enterocolitis

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The pathogenesis of necrotizing enterocolitis (NEC) is poorly understood. We have established several animal models of NEC by using a combination of various stimuli and stress, including endotoxin, PAF, TNF, and hypoxia. We discuss the mechanism of their actions and the possible roles of these factors in the pathogenesis of human NEC. *Hypoxia, necrotizing enterocolitis, platelet activating factor, tumor necrosis factor*

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The etiology and pathogenesis of necrotizing enterocolitis (NEC) are unclear (1). NEC occurs as isolated cases endemically in neonatal nurseries, but sometimes epidemic clusters of cases are seen. The latter occurrence is thought to be caused by infectious agents, although repeated attempts have failed consistently to identify a specific bacteria or virus. Although no definitive etiology has been identified, epidemiological studies point to prematurity (1), infection (2), oral feeding (3), and hypoxia (4) as important risk factors for the development of NEC. We have investigated the role of some of these factors in the pathogenesis of NEC in experimental animals as well as in clinical studies.

Animal model 1: LPS, PAF and TNF-induced bowel necrosis

Our first model of bowel necrosis was established in rats and mice by injection of endotoxin (lipopolysaccharide, LPS) (5), PAF (platelet-activating factor, paf-acether) (6, 7), tumor necrosis factor-α (TNF, cachectin) (8), or a combination of these agents. The rationale for using these agents was as follows: (a) If the infectious agent which causes NEC is bacterial, resident intestinal flora such as *E. coli* and its toxin product, LPS, would be a highly probable candidate. Previous investigations have shown that oral feeding markedly increases the growth of *E. coli* in the intestinal tract (9), and NEC usually develops following oral feeding. (b) Injection of LPS induces endogenous production of lipid mediators (10, 11) and cytokines (11–13). Among these mediators, PAF and TNF seem to be the most important ones, because administration of PAF (reviewed in refs. 14–16) or TNF (17, 18) to animals mimics symptoms and signs of shock, and pretreatment of the animal with anti-TNF (18) or PAF antagonists (16, 19) prevents LPS-induced shock and increases survival.

PAF is an endogenous phospholipid mediator produced by inflammatory cells, platelets, and endothelial cells (reviewed in refs. 14, 15, 20). In addition, bacteria such as *E. coli* have been reported to be able to synthesize PAF (21, 22). The main source of TNF is monocytes and macrophages (12, 23), although lymphocytes (23, 24) and other tissues (25) have also been shown to produce this cytokine upon stimulation.

Administration of LPS (2–5 mg/kg) alone induces a slow hypotensive response with mild intestinal necrosis in rats (5). The effects of PAF on blood pressure and the intestinal tract are more rapid and dramatic. PAF is probably the most potent agent that induces intestinal injury. In our hands, a dose as small as 1.5 μg/kg often causes necrosis of the small intestine of varying degree in the rat in 2 h (26). Since rat platelets are refractory to PAF (6, 27) the development of necrosis is independent of the thromboembolic effect of PAF. The necrosis produced is usually focal (Fig. 1) in the jejunum or ileum, although more frequently in the distal ileum. When a high dose (> 3 μg/kg) is injected into the circulation, the entire small bowel may be affected. Histologically, the necrosis begins at the tip of the villi (6), later extends to the entire mucosa (Fig. 2A), and may become transmural (Fig. 2B) if the dose is sufficiently high. The effects of PAF and LPS are synergistic (Table 1) (6, 7, 28). Furthermore, LPS-induced bowel injury could be blocked by pretreatment with PAF antagonists (5), suggesting that this effect is mediated by endogenous PAF production.

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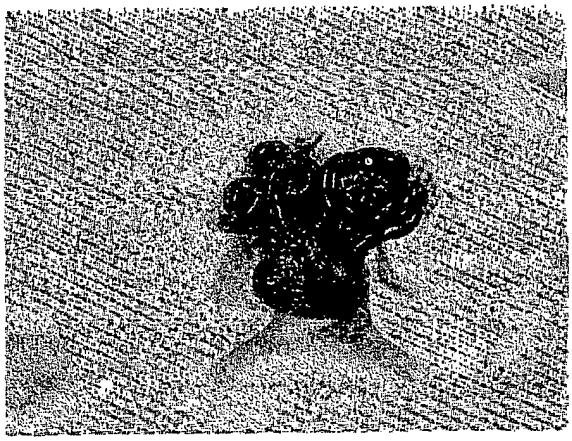

Fig. 1. Development of segmental small bowel necrosis 1 h following injection of PAF (2 μg/kg, iv) to a Sprague-Dawley rat (approx. 100 g body weight).

Systemic administration of TNF (1 mg/kg) also induces hypotension and mild intestinal injury in rats (8). When TNF (0.5 mg/kg) was combined with a small dose of LPS (200 μg/kg), profound shock and severe intestinal necrosis developed (8). This synergism is also observed in mice (29, 30). Complement activation probably plays a role in TNF/LPS-induced or PAF-induced bowel injury, since C5 deficient mice are protected from injury (29, 30). Furthermore, injection of TNF/LPS results in endogenous PAF production, which may in turn activate the complement system. This is inferred from our observations that PAF administration causes complement activation in vivo (30), and pretreatment with a PAF receptor antagonist protects mice from TNF/LPS induced shock, complement activation, intestinal injury and death (Table 1) (30).

Mechanism of PAF-induced bowel injury: roles of leukotrienes, catecholamines, PMNs, oxygen radicals, TNF and endogenous PAF

PAF has a short half-life in the circulation, due to the high plasma and tissue acetylhydrolase activity (31, 32), which rapidly degrades PAF into the biologically inert lyso-PAF. However, the in vivo action of PAF is prolonged. Furthermore, PAF is a vasodilator (33), while at high dose its effect on the mesenteric vascular bed is protracted vasoconstriction (33, 34). One of the explanations is that secondary mediators with sustained splanchnic vasoconstricting action are released after PAF administration. Indeed, we have shown that leukotriene C4 (35) and norepinephrine (36), both having potent splanchnic vasoconstriction effect, are released after PAF injection. Moreover, in vivo administration of leukotriene C/D antagonists (7, 34), or alpha blockers (34), did not reverse shock, but prevented PAF-induced intestinal injury (Table 2). The cytotoxicity effect of PAF is most likely due to reactive oxygen radical formation, since PAF-induced bowel necrosis could be ameliorated by infusion of superoxide dismutase and catalase (37), or allopurinol (37), a xanthine oxidase inhibitor (Table 2).

Another mechanism which may account for the prolonged action of PAF is that PAF not only induces TNF formation in the intestine and liver (38), but also induces its own production in vivo (26). This is demonstrated by the observation that PAF antagonists decreased PAF-induced PAF production in the intestine (Table 2) (5, 26).

The source of secondary mediators is unknown. It is possible that catecholamine is released from the intestinal tissue. Other mediators, such as peptide leukotrienes, oxygen radicals, and endogenous PAF are likely to originate, at least in part, from the resident or infiltrating inflammatory cells. Although it is well established that

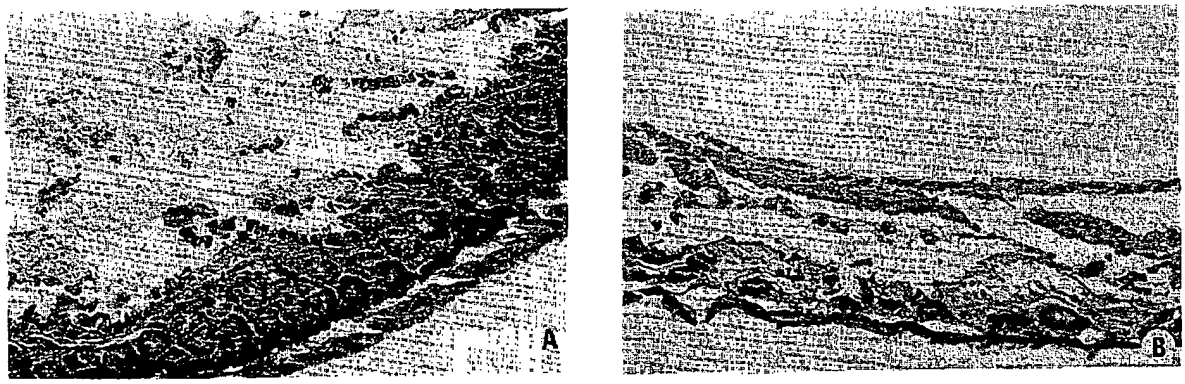

Fig. 2. Microscopic appearance of the small intestine from a rat injected with PAF (2 μg/kg) showing necrosis of the mucosa (A) and transmural necrosis (B).

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Table 1. Effects of PAF, LPS and TNF on systemic blood pressure, hematocrit, and intestinal injury.

Agent (mg/kg)	Species (No. animal)	End B.P.* (mmHg)	Hct	% Gut perf.	% Gross Nec.	Ref.
PAF[a] (.06)	Rat (7)	26 ± 5	71 ± 4	32 ± 6	NR	7
LPS (.6) + PAF[a] (.03)	Rat (11)	44 ± 13	69 ± 5	48 ± 16	NR	7
SRI[c] + LPS (.6) + PAF[a] (.03)	Rat (5)	104 ± 7	49 ± 2	100	0	28
PAF[b] (.015)	Rat (11)	43 ± 10	62 ± 1		60 ± 10	26
WEB[e] (1) – PAF[b] (0.15)	Rat (6)	103 ± 11	50 ± 1		0	26
TNF (1)	Rat (3)	52 ± 14	43 ± 1		17 ± 8	8
TNF (0.5)	Rat (6)	88 ± 8	44 ± 1		0	8
TNF (0.5) + LPS (.2)	Rat (5)	20 ± 5	46 ± 3		35 ± 7	8
SRI[c] + TNF (.5) + LPS (.2)	Rat (3)	62 ± 11	47 ± 2		0	8
PAF[b] (.03)	Mouse (15)	31 ± 3	67 ± 2	10 ± 4[#]	NR	30
TNF (0.2) + LPS (3)	Mouse (11)	20[##]	70 ± 3	9 + 6	NR	29
SRI[d] + TNF (.2) + LPS (3)	Mouse (6)	59 ± 2		95 ± 2	0	30

* The experiment usually lasts 2 h.
NR: All animals have gross necrosis. % of intestinal length necrotic not recorded.
PAF[a]: 1-alkyl-2-acetyl-phosphorylcholine used in earlier work may contain mixture of different molecular species of PAF.
PAF[b]: Pure 1-hexadecyl-2-acetyl-phosphorylcholine was used in these studies.
SRI[c]: SRI 63–119 (3 mg/kg), PAF antagonist (gift from Sandoz Research Institute, E. Hanover, NJ, USA).
SRI[d]: SRI 63–441 (5 mg/kg, iv), PAF antagonist (gift from Dr D Handley, Sandoz Research Institute).
WEB[e]: WEB 2086, PAF antagonist (gift from Boehringer Ingelheim, Mainz, Germany).
Only 3 mice survived at the end of experiment.
Only 1 animal survived at the end of experiment.

macrophages (39), mast cells (40, 41) and endothelial cells (39, 42, 43) are capable of elaborating these mediators, virtually nothing is known about the roles of these cells in the intestine during endotoxin shock or infection. Infiltrating PMNs may play an important role in mediating tissue injury, probably via release of oxygen radicals. This is suggested by the observation that depletion of PMNs by vinblastine prevents PAF-induced or PAF/LPS-induced intestinal injury in rats (44) and mice (30). Another line of evidence is that injection of anti-CD18 (adhesion molecule on PMNs) also prevents the PAF-induced increased endothelial (45) and mucosal (46) permeability. Another potentially important cell in intestinal injury may be Paneth cells. These cells have been shown constitutively to express low levels of TNF (47). Although the mechanism of TNF induction in the bowel is unclear, we have observed increased TNF gene expression in the Paneth cells, lamina propria eosinophils and infiltrating (but not resident) macrophages in infants with NEC during the acute stage (Fig. 3) (48).

Animal model 2: role of hypoxia

Hypoxia is one of the major risk factors for NEC, and animal studies in the early 1970s confirmed that both acute asphyxia and prolonged hypoxic exposure resulted in mild mucosal necrosis in animal models (4, 49). However, the pathophysiology of hypoxia-induced bowel injury is not well understood. We found that acute hypoxia (100% N_2, 2 min) and moderate hypoxia (10% O_2, 30 min) in weanling rats increased circulating PAF concentrations (13.8 ± 2.9 and 41.1± 11.7 ng/ml

Table 2. List of drugs that prevent or ameliorate PAF-induced intestinal necrosis in rats.

Agent	Dose (mg/kg)	Mechanism	Ref.
FPL 55712	5	LTC4/D4 antagonist	7
ICI 198615	10–20	LTC4/D4 antagonist	34
Phenoxybenzamine	20	alpha blocker	34
SOD + catalase	@10*	oxygen radical scavenger	37
Allopurinol	5	xanthine oxidase inhibitor	
WEB 2086	1	PAF antagonist, also blocks endogenous PAF production)	26
PGE1	0.27*	vasodilation, cytoprotect. inhibits norepinephrine	34
Vinblastine	0.75	PMN depletion	30,44

* Slow iv infusion.

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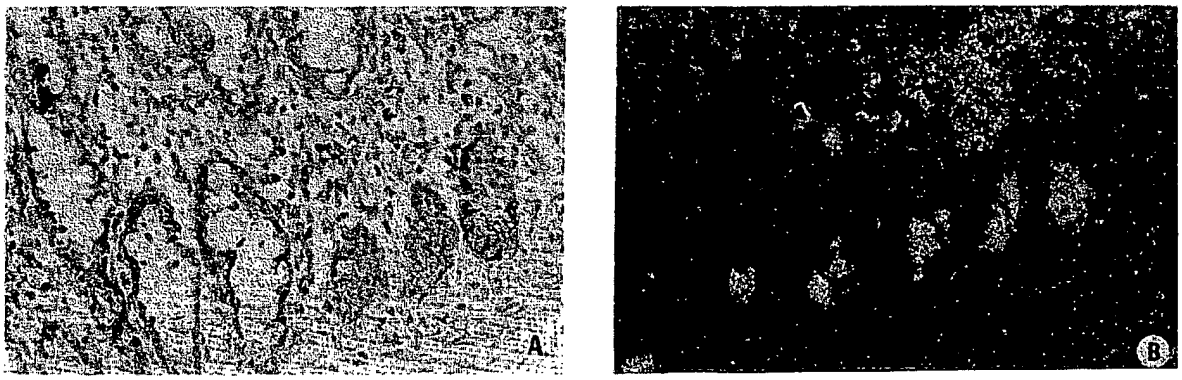

Fig. 3. In situ hybridization study showing a marked increase of TNF mRNA in Paneth cells from a patient with NEC during the acute stage. Light field (A) and dark field (B).

respectively) compared to control animals (2.1 ± 0.8 ng/ml) (50). Furthermore, pretreatment with PAF antagonist WEB 2086 or SRI 63 441 prevents hypoxia-induced mucosal injury (50). These findings suggest a role of PAF in hypoxia-induced intestinal injury.

However, the above protocol produces only mild mucosal necrosis quite dissimilar from human NEC. In order to further approximate the human disease, we studied the combined effects of LPS and hypoxia on PAF metabolism and intestinal injury (51). Combining low dose LPS (2 mg/kg, iv) and moderate hypoxia (5% O_2, 90 min) together, it is possible to induce hypotension, acidosis, mesenteric vasoconstriction, increased intestinal PAF content, and significant bowel necrosis. Furthermore, these morbidities were all attenuated by PAF receptor blockade (Fig. 4). These experiments suggest that hypoxic stress and bacterial products may synergize to cause intestinal injury via PAF production. The cell source of PAF in hypoxic stress is probably endothelium. This is

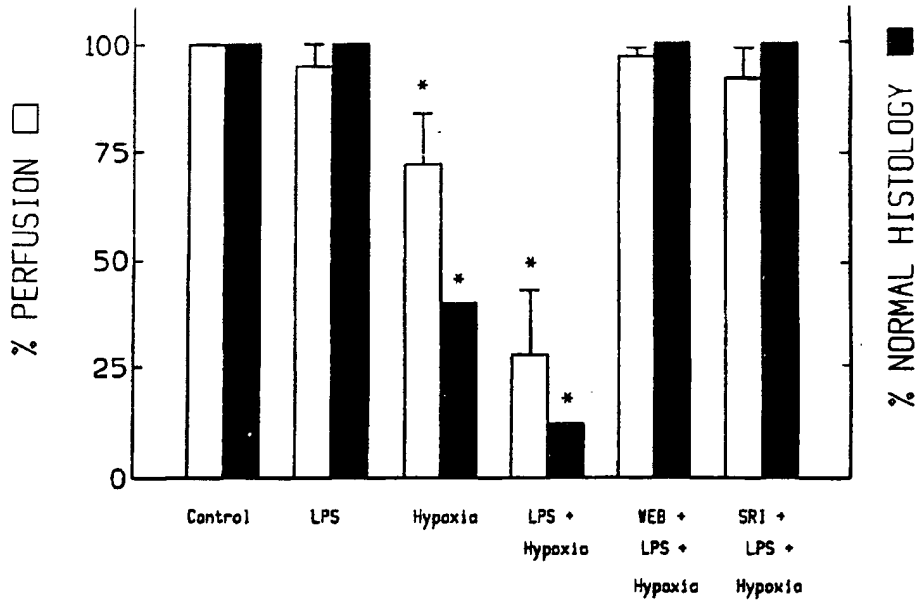

Fig. 4. Synergistic effect of LPS (2 mg/kg) and hypoxia (5% O_2, 90 min) on small intestinal hypoperfusion and injury in the rat, and prevention by PAF antagonists. (* $p < 0.05$ compared to control). The open bars depict percentage of intestinal perfusion at 3 h using the Evans blue method (7). The filled bars represent percentage of animals with normal histology or minimally abnormal histology in each group. PAF antagonists: WEB 2086 (1 mg/kg, iv) and SRI 63-441 (5 mg/kg, iv).

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suggested by our observation that hypoxia (1% O_2) enhances PAF production by cultured human endothelial cells (52).

Role of PAF and TNF in human NEC

Despite the substantial evidence supporting the role of PAF and TNF in animal models of bowel necrosis, their role in human NEC remains speculative. To investigate the association between PAF and neonatal NEC, we measured plasma samples from NEC patients and compared them with age-matched control patients with other diseases (53). We found that NEC patients had higher PAF levels (18.1 ± 3.6 vs 3.1 ± 0.9 ng/ml, $p < 0.01$), higher TNF concentrations (136 ± 75 vs 1.5 ± 0.8 U/ml, $p < 0.05$), and lower acetylhydrolase (PAF degrading enzyme) activity (10.6 ± 0.7 vs 23 ± 1.4 nmol/ml/min, $p < 0.01$), than control premature infants. Although these measurements were obtained from severely affected NEC patients, they confirmed an association between circulating PAF, TNF and NEC. In order to investigate the possibility of earlier changes in circulating PAF levels in premature infants at risk for NEC, we prospectively measured plasma PAF and endotoxin in premature infants before and after enteral feeding (a risk factor for NEC), and upon the development of suspected or confirmed NEC (54). We found that significantly more patients had detectable PAF levels after feeding than before feeding (26% vs 7%, $p < 0.05$). Although patients with suspected NEC had similar PAF results compared to controls, infants with proven NEC had markedly elevated PAF levels. These observations confirm that elevated plasma PAF levels are associated with NEC, and suggest that enteral feeding may increase the risk of NEC by increasing circulating PAF.

Since older children and adults do not develop NEC, we hypothesized that PAF metabolism may be different in neonates. We found that serum acetylhyrolase activity was lower for neonates than older children and adult controls (8.2 ± 1.4 vs 30 ± 1.6 nmol/ml/min, $p < 0.01$), and that the enzyme activity increases linearly with the natural logarithm of age from 0 to 6 weeks when it reaches adult values (55). These results suggest that the decreased ability to degrade circulating PAF may increase the risk of neonates to PAF-related diseases such as NEC.

Proposed mechanism for the pathogenesis of NEC

Our hypothesis for the pathogenesis of NEC is as follows: The initial insult could be hypoxia or a mild viral or bacterial infection which results in mild mucosal damage. Following the enteral feeding, the intestinal flora proliferate and may gain entry into the intestinal tissue due to previous mucosal damage and immaturity of the "mucosal barrier", eliciting endogenous production of PAF and TNF. (PAF may also be contributed by the gut bacteria.) If the acetylhydrolase is deficient, PAF may accumulate, synergize with TNF and LPS, reaching the threshold necessary to trigger a cascade of PMN activation, complement activation, and release of oxygen radicals. As a result, focal bowel necrosis develops, which further facilitates bacteria entry, thereby launching a self-perpetuating vicious cycle, resulting in sepsis, shock, and sometimes, death.

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